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Sclerostin deficiency modifies the development of CKD-MBD in mice

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Abstract

Sclerostin is a soluble antagonist of canonical Wnt signaling and a strong inhibitor of bone formation. We present experimental data on the role of sclerostin in chronic kidney disease - bone mineral disorder (CKD-MBD).

Methods: We performed 5/6 nephrectomies in 36-week-old sclerostin-deficient ($SOST^{-/-}$) B6-mice and in C57BL/6J wildtype (WT) mice. Animals received a high phosphate diet for 11 weeks. The bones were analyzed by high-resolution micro-computed tomography (μ CT) and quantitative bone histomorphometry. Aortic tissue was analyzed regarding the extent of vascular calcification.

Results: All nephrectomized mice had severe renal failure, and parathyroid hormone was highly increased compared to corresponding sham animals. All $SOST^{-/-}$ animals revealed the expected high bone mass phenotype. Overall, the bone compartment in WT and $SOST^{-/-}$ mice responded similarly to nephrectomy. In uremic WT animals, μ CT data at both the distal femur and lumbar spine revealed significantly increased trabecular volume compared to non-uremic WT. In $SOST^{-/-}$ mice, the differences between trabecular bone volume were less pronounced when comparing uremic with sham animals. Cortical thickness and cortical bone density at the distal femur decreased significantly and comparably in both genotypes after 5/6 nephrectomy compared to sham animals (cortical bone density -18% and cortical thickness -32%). Overall, 5/6 nephrectomy and concomitant hyperparathyroidism led to a genotype-independent loss of cortical bone volume and density. Overt vascular calcification was not detectable in either of the genotypes.

Conclusion: Renal osteodystrophy changes were more pronounced in WT mice than in $SOST^{-/-}$ mice. The high bone mass phenotype of sclerostin deficiency was detectable also in the setting of chronic renal failure with severe secondary hyperparathyroidism.

Introduction

The term ‘bone-vascular calcification paradox’ describes various disease states with dysregulated calcification processes, characterized by the coincidence of skeletal demineralization/hypomineralization and vascular calcification. Chronic kidney disease - mineral and bone disorder (CKD-MBD) is a prototypic condition for this paradoxical calcification ⁽¹⁾ and encompasses three major disturbances associated with CKD: bone disease, cardiovascular disorders, and dysregulated mineral metabolism ^(2,1). Wnt signaling appears to be a major player in the crosstalk within the bone-vascular axis since it regulates both vascular development and endothelial cell specification, as well as bone modeling and remodeling ^(3,2,4). Secreted Wnt inhibitors, such as sclerostin, are a group of proteins which bind extracellularly to the transmembrane receptor as Wnt ligands. Sclerostin is a member of the cystine knot family and a product of the SOST gene ⁽⁵⁾. This glycoprotein (22 kDa) is secreted almost exclusively by osteocytes and to a lesser extent by other cell types including osteoclast precursors and renal and vascular cells ⁽⁶⁾.

Given the important role of Wnt signaling in the biology of bone and vasculature, it is tempting to speculate about the potential role of Wnt signaling in all three aspects of CKD-MBD ⁽⁷⁾. *First*, sclerostin is involved in the early development of murine, renal osteodystrophy ⁽⁸⁾. Moreover, according to its physiological function, preliminary human data point towards an association between high levels of sclerostin and low bone turnover in end-stage renal disease (dialysis) patients ⁽⁹⁾. *Second*, Wnt signaling plays a role in human atherosclerosis ⁽¹⁰⁾, and missense mutations in LRP6 are associated with premature, coronary artery disease and osteoporosis ⁽¹¹⁾. While participation of Wnt signaling in atherosclerosis of the general population has already been extensively reviewed ⁽¹⁰⁾, the potential role of Wnt signaling

and sclerostin in uremia-associated calcifying vascular disease is a novel, evolving field. *Third*, sclerostin modulates PTH action ⁽¹²⁾ and hence interacts with mineral metabolism in CKD-MBD.

Taken together, the sclerostin metabolism is linked to the entire spectrum of CKD-MBD. In the present study, we evaluated the potential role of an absence of sclerostin in CKD-MBD using a novel *in vivo* approach: we investigated the bone-vascular axis phenotype in sclerostin-deficient mice with normal renal function which are characterized by a high bone mass phenotype in the absence of CKD ⁽¹³⁾, as well as in mice with additional induction of chronic renal failure.

2. Methods

2.1. Animal experiments

Thirty-five to thirty-eight-week-old, female B6-SOST^{tm1Dgen} mice⁽¹²⁾ with corresponding wildtype (WT) controls, underwent one-step 5/6 nephrectomy (5/6 Ntx) (12 animals in each group, mean body weight WT 25.7 g, SOST^{-/-} 31.2 g) or random sham surgery. The surgery was performed under 2.5% isoflurane anesthesia on a 37°C warming plate. After flank incision, the left kidney mass was reduced to one third. The contralateral kidney was removed completely. Analgesics were given for 3 days after surgery (Buprenorphine, Essex Pharma, Munich, Germany). After one week of recovery, the mice were fed a high (1.65%) phosphate diet (AB diets, Woerden, the Netherlands). The mice were sacrificed 12 weeks after surgery. Seven days before sacrifice, the mice were injected with tetracycline (10 mg/kg) and at -3 days with doxycycline (25 mg/kg) subcutaneously. At the end of the experiment, blood pressure was measured by the tail vein occlusion method (Kent scientific, Torrington, CT, USA). Finally, blood was collected by puncturing the left ventricle for exsanguination. The organs were harvested and snap frozen in liquid nitrogen. The femur, calvaria and vertebra were stored in 70% ethanol.

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines. The study was approved by the national institution LANUV (Permit number 2014.A326). Animals were housed in the Institute of Laboratory Animal Science of the RWTH Aachen with free access to food and water and in a 12 h day and night cycle.

2.2. Blood

Blood was collected and serum was obtained by centrifugation of whole blood at 2000 g for 10 min at 4°C. PTH and FGF23 were measured in serum using ELISAs (Immutopics, CA,

USA; Tecomedical, Bünde, Germany); urea and creatinine were measured following routine clinical procedures.

2.3 Calcium content

Calcium in aorta, carotid artery, heart and kidney was quantified by the o-cresolphthalein method (Randox Laboratories, Crumlin, UK) and by von Kossa staining, as in ⁽¹⁴⁾. For total tissue calcium, tissue sections were weighed and calcium was mobilized by 10% formic acid. The absorption was measured at 550 nm on a Tecan Sunrise microplate absorbance reader (Tecan, Mennedorf, Switzerland). Final total tissue calcium is expressed as mg/g wet weight. Briefly, von Kossa staining was performed in paraffin-embedded tissues. Sections of 4µM were immersed in 1% aqueous AgNO₃ (Merck, Darmstadt, Germany), followed by 5% NaHCO₃ (Merck, Darmstadt, Germany) and 9.25% formalin (Merck, Darmstadt, Germany). Sections were developed using 5% aqueous sodiumthiosulfate (Merck, Darmstadt, Germany) ⁽¹⁴⁾.

2.4 Bone histomorphometry

Femur samples were fixed in 70% ethanol and processed for histomorphometric analysis as described previously ⁽¹⁵⁾. In summary, samples were dehydrated in increasing ethanol concentrations, followed by ethyl acetate. After dehydration, samples were embedded in methylmethacrylate under N₂ atmosphere. Five micrometer sections of the embedded bone were cut and stained using a modified Goldner trichrome stain. Ten micrometer sections were mounted unstained using 100% glycerol for fluorescence microscopy of the tetracycline labels. In mouse tibia, the trabecular bone becomes less dense towards the diaphysis. In order to standardize the location of the measured fields and avoid analysis of the secondary spongiosa and the cortical bone, at least one field of separation is kept between the measured fields and the cortical bone and the growth plate. The entire width of the bone sample is

measured with a minimum of seven adjacent fields (approx. 1.2 mm²). If the sample size is too small to obtain the required number of fields, a second section is analyzed. Quantitative histomorphometric analysis was performed using a semi-automatic image system (Axiovision v 4.51, Zeiss, Germany), running a custom program. The digital camera, connected to the microscope, allows images to be captured directly from the microscope. These images are then displayed and measured separately on the Goldner-stained sections and the unstained tetracycline-labeled sections. Before each measurement cycle, calibration of image pixel size is performed using a calibration grid. The average pixel size of the horizontal and vertical camera direction is used for calibration of the results. The primary measurements obtained are saved electronically and used to calculate the secondary parameters. These parameters have been reported in detail ^(16,17). The Goldner-stained sections are used to measure the static bone parameters. On the digital image, the mineralized area and the osteoid area are drawn manually by the operator. The regions of erosions and the presence of osteoblasts and osteoclasts are marked as well and the system then calculates the respective areas and perimeters.

The mounted unstained sections are measured in a similar way. The labels are drawn on the computer screen, and the program then calculates the distance between the labels at regular intervals perpendicular to the labels. The single and double-labeled perimeter and total perimeter are also measured after the operator has drawn these on the computer ^(16,17). All results are reported as two-dimensional measurements using establish nomenclature ⁽¹⁸⁾.

2.5 Micro-computed tomography

High-resolution micro-computed tomography (μ CT) analyses of the femur and lumbar spine were performed *ex vivo* using a μ CT40 instrument (Scanco Medical AG, Switzerland) at an isotropic nominal resolution of 6 μ m (femur) or 8 μ m (spine), respectively. The following settings were applied to all measurements: integration time: 200 ms, 70 E(kVp); 114 μ A. For

determination of bone structural parameters, a segmentation threshold of 240 and a Gaussian filter ($\sigma = 0.7$, support of one voxel) were used to segment the mineralized bone tissue. Then, 0.63 mm of the distal femur metaphysis, 0.48 mm of the femur diaphysis and 0.42 mm of lumbar vertebra L3 were analyzed, respectively.

2.6 Real-time PCR

The TaqMan® gene expression assay was applied on cDNA obtained from aortic tissue. The qPCR Core Kit for SYBR Green (Eurogentec, Liège, Belgium) was applied for a two-step quantification on a Real-Time PCR TagMan® system. GAPDH was used as a housekeeping gene. The following primers were used: Cbfa1: sense: caagtagccaggttcaacgatct, anti-sense: gacttgatggtcaaggtgaaactctt; α -SMA: sense: GTCCCAGACATCAGGGAGTAA, anti-sense: TCGGATACTTCAGCGTCAGGA; GAPDH: sense: AGATGGTGATGGGCTTCCC, anti-sense: GGCAAATTCAACGGCACAGT.

2.7 Statistics

Differences between treatment groups were assessed by Kruskal-Wallis test followed by Mann-Whitney test with Bonferroni's multiple comparison. Statistical significance was defined as $p < 0.05$, and highly significant $p < 0.001$. Statistical power analysis was performed with a two-sample t-test with unequal sigma (Piface 1.72).

3. Results

3.1 Induction of CKD

The mortality rate up to the end of the experiment was 33% (4 of 12) for 5/6 Ntx WT and 25% (3 of 12) for 5/6 Ntx SOST^{-/-} mice. The mean survival time was not statistically different between WT and knockout animals. The 5/6 Ntx increased serum creatinine and urea levels significantly and to the same extent in both strains and the various subgroups (Figures 1a and 1b). The final body weight was statistically lower in WT sham animals ($24.7 \text{ g} \pm 2.0$) compared to SOST^{-/-} ($29.0 \text{ g} \pm 3.6$), and decreased after 5/6 Ntx in both groups ($24.0 \text{ g} \pm 1.3$ in WT and $25.4 \pm 3.2 \text{ g}$ in SOST^{-/-}). The 5/6 Ntx further induced a tremendous increase in serum parathyroid hormone (PTH) in WT mice (7-fold) and in SOST^{-/-} mice (16-fold), which was highly significant compared to their respective sham controls (Figure 1c). Additionally, nephrectomy induced a significant increase in FGF23 in both Ntx groups compared to their corresponding sham controls (WT 29-fold, SOST^{-/-} 18-fold), lacking significance between the strains (Figure 2).

There was no difference in systolic blood pressure between sham WT ($121.3 \pm 7.8 \text{ mm Hg}$) and sham SOST^{-/-} (112.6 ± 19.7) animals at the end of the experiments. In the 5/6 Ntx groups, systolic blood pressure was significantly higher in WT compared to SOST^{-/-} mice (132.1 ± 20.5 vs $87.9 \pm 12.1 \text{ mm Hg}$).

3.2 Cardiovascular and extra-skeletal calcification

On the von Kossa-stained sections, calcifications were found in the aortic heart valves and inside the myocardium in some animals in all groups (Figure 3), however, no statistically significant differences were found by analyzing the von Kossa-positive area. Few calcifications were found on von Kossa-stained sections of the descending aortas, aortic arch and myocardium of all groups, and no differences were observed between the groups.

Quantification of total kidney and carotid artery calcium revealed no statistically significant differences between groups (Figure 4a-b). Variable calcifications occurred in the kidneys of animals that underwent 5/6 Ntx, leading to non-significantly higher concentrations of renal calcium in those animals. $SOST^{-/-}$ mice that underwent 5/6 Ntx had significantly higher calcium in the myocardium compared to sham knockout mice (Figure 4c). Calcium in aortic tissue was undetectable.

3.3 Transdifferentiation of aortic tissue

Aortic expression of *Cbfa1* mRNA was significantly increased only in 5/6 Ntx WT mice, compared to all other groups (Figure 5). No statistical differences could be observed for α -SMA mRNA expression between the different groups (data not shown).

3.4 Bone histomorphometry

Quantitative bone histomorphometric analysis of static and dynamic bone parameters in the femora is presented in Figures 6 and 7. Figure 8 shows representative microscopic pictures of Goldner-stained sections of the various groups studied.

Irrespective of surgery, femora of all $SOST^{-/-}$ mice had significantly higher bone area and mineralized area expressed per total tissue area, eroded perimeter and osteoclast perimeter (expressed per bone perimeter, mineral apposition and bone formation rate, trabecular number and thickness and bone marrow fibrosis compared to corresponding WT controls. This high bone turnover-high bone mass phenotype of the $SOST^{-/-}$ mice is also evidenced by the microscopic picture in the Goldner-stained sections (Figure 8). With regard to osteoblasts, no significant differences were seen in osteoblast perimeter expressed per bone perimeter between WT and $SOST^{-/-}$ mice nor between 5/6-Ntx and shams.

Regarding 5/6-Ntx, SOST deficiency associated with a significantly increased eroded perimeter, osteoclast perimeter, mineral apposition rate as well as trabecular number and more severe marrow fibrosis compared to sham-operated animals (Figure 6).

3. 5 Micro CT

Irrespective of renal status μ CT analysis confirmed the strong bone phenotype of SOST-deficiency: The trabecular phenotype of the distal femur metaphysis was complemented by the bone histomorphometric analysis (Figure 9, left). Cortical thickness was 28% significantly lower in WT Ntx versus sham mice (WT Ntx 0.077 ± 0.006 mm; WT sham 0.108 ± 0.012 mm) and 35% lower in SOST^{-/-} Ntx versus sham mice (SOST^{-/-} Ntx 0.103 ± 0.015 mm; SOST^{-/-} sham 0.158 ± 0.019 mm). Cortical bone mineral density was 17% significantly lower in WT Ntx compared to sham mice (WT Ntx 793.9 ± 39.6 mg/ccm; WT sham: 951.7 ± 28.7 mg/ccm) and 18% lower in SOST^{-/-} Ntx versus sham mice (SOST^{-/-} Ntx 894.2 ± 97.2 mg/ccm; SOST^{-/-} sham 1084.7 ± 50.5 mg/ccm) (Figure 9, left).

The effect of 5/6 Ntx was less pronounced in the femur diaphysis. The decrease in cortical thickness (-18% in WT, sham: 0.171 ± 0.011 , Ntx: 0.141 ± 0.004 , and -8% in SOST^{-/-}, sham: 0.249 ± 0.023 , Ntx: 0.230 ± 0.022) and cortical bone mineral density (-4% in WT, sham: 1183.1 ± 14.6 , Ntx: 1137.0 ± 12.8 , and -1% in SOST^{-/-}, sham: 1290.3 ± 22.7 , Ntx: 1276.3 ± 13.9) was only statistically significant in the WT (Figure 9, middle).

In the lumbar spine (Figure 9, right) there was a significant increase in trabecular bone volume/tissue volume (+112% in WT, sham: 14.6 ± 1.9 , Ntx: 31.1 ± 5.9 , +37% in SOST^{-/-}, sham: 47.0 ± 6.6 , Ntx: 64.3 ± 7.2) and cancellous bone mineral density (+85% in WT, +24% in SOST^{-/-}) in the 5/6-Ntx versus shams.

4. Discussion

Sclerostin substantially regulates bone mineralization processes and is a potent anti-anabolic agent in the skeleton. While reduced activity of sclerostin *in vivo* contributes to increased bone mass and strength (“high bone mass phenotype”) ^(19,12), the opposite is true in experimental models with overexpression of sclerostin ^(5,20). There are convincing reasons to believe that sclerostin actively participates in CKD-MBD, as it appears to be involved in the development of renal osteodystrophy, as well as in uremic cardiovascular disease ^(21,20,21). Hence, the present experiments were developed to further elucidate the role of Wnt signaling in CKD-MBD. The major findings of this study are as follows: *first*, uremia, accompanied by severe renal hyperparathyroidism, did not substantially affect the strong bone phenotype induced by sclerostin deficiency. *Second*, we found that an absence of sclerostin did not associate with the development of relevant amounts of vascular calcification in the 5/6 Ntx model, an important finding in regard to the potential effects of sclerostin blockade in vascular disease ⁽²²⁾.

Overall, the present experiments significantly expand previously published findings from a similar mouse model by Cejka et al. ⁽²⁰⁾. There are three major differences between the previous experiments and the present study. In the previous experiments, the animals were not on a high phosphate diet, severe secondary hyperparathyroidism was not induced and there was no report on the vascular phenotype of their 5/6 Ntx $SOST^{-/-}$ mice. Also, Cejka et al. demonstrated only minor skeletal differences between uremic $SOST^{-/-}$ and WT mice similar to our study, but, in contrast to our findings, secondary hyperparathyroidism was absent ⁽²³⁾. Compared to Cejka’s study, the effect of SOST depletion on the bone area (volume) was much more pronounced (in our study a 10-fold versus 2-fold increase) as was the increase in bone formation rate (10-fold versus 2-fold). Finally, in addition to increased PTH levels, we also observe increased bone marrow fibrosis after 5/6 nephrectomy which points towards secondary hyperparathyroidism induced osteitis fibrosis. To the best of our knowledge, no data are currently available in the literature linking sclerostin with the development of fibrosis

in the bone. However, at least in the kidney, SOST gene deletion has been reported to accelerate the progression of renal interstitial fibrosis in obstructive kidney injury ⁽²⁴⁾.

In regard to sclerostin's physiological skeletal functions, speculations about similar anti-mineralization effects in the vasculature may arise ⁽²²⁾. Interestingly, sclerostin deficiency did not induce soft tissue calcification in our 5/6 Ntx model. While calcium content in the myocardium and the carotid arteries was indeed higher in some uremic animals with sclerostin-deficiency compared to uremic WT mice, an overall tendency to develop cardiovascular calcification was apparently absent, considering the low levels of Cbfa1 in SOST-deficient aortic tissue. Cbfa-1 upregulation is considered to be a master switch in vascular calcification development ⁽²⁵⁾. In summary, our data does not allow drawing definite conclusions about a potential mechanistic link between absence of SOST and calcification. Further investigations will be required in order to determine why we observed increased calcification inside the myocardium of nephrectomized SOST^{-/-} mice, which is not associated with calcification of resident vessels. We need to acknowledge, however, that our CKD animal model is suboptimal to investigate this potential association, since we failed to induce overt aortic calcifications also in the uremic WT mice. One reason for this failure is presumably the calcification resistant genetic C57/BL6 background which requires additional calcification stimuli (e.g. treatment with high vitamin D doses) ^(26,27). Moreover, we analyzed two markers related to vascular calcification: α -SMA and Cbfa1. We failed to induce a loss of α -SMA in our model, but the early marker Cbfa1 was already significantly increased in Ntx WT, underlining a gain of osteogenic phenotype cells inside the aorta ⁽²⁵⁾.

PTH downregulates sclerostin expression in osteocytes, and this interaction represents an important aspect of how PTH stimulates bone metabolism ⁽²⁸⁾. Kramer and co-workers convincingly showed that skeletal PTH actions rely upon sclerostin physiology ⁽²⁹⁾. They investigated the effects of intermittent PTH administration in mouse models with either

sclerostin overexpression or sclerostin deficiency. In both genetically engineered mouse models, the response to intermittent PTH treatment in terms of stimulation of bone metabolism was significantly diminished. Therefore, the authors came to the conclusion that suppression of sclerostin in osteocytes is necessary to mediate anabolic responses to PTH⁽²⁹⁾. Interestingly, WT and SOST-deficient uremic mice developed a comparable degree of secondary hyperparathyroidism, arguing against upregulation of sclerostin being a stand-alone stimulator for biochemical hyperparathyroidism. Bone formation rate was dramatically increased in both SOST^{-/-} mice groups (with and without uremia), irrespective of the huge difference in PTH levels between both groups. This indicates that sclerostin, rather than PTH, was the crucial regulator of bone formation. It should be noted, however, that despite the highly comparable bone formation rates, fibrosis was much more severe in the uremic SOST^{-/-} mice compared to WTs. Thus, the secondary hyperparathyroidism aggravated the development of fibrosis in SOST-depleted mice, irrespective of the amount of bone turnover. As expected for a uremic milieu, and in accordance with PTH, FGF23 serum levels also rose significantly in both experimental 5/6 Ntx subgroups. The limited number of animals precludes conclusions about the influence of SOST deficiency upon the specific degree of hyperparathyroidism (numerically lower in SOST^{-/-}) and “hyper-FGF23-emia” (numerically higher in SOST^{-/-}) compared to uremic WT animals. The μ CT data indicate that WT and SOST^{-/-}, at least in the presence of a high phosphate diet, responded similarly to 5/6 Ntx in the trabecular and cortical bone compartment of the femur. Additionally, comparable results were obtained in the axial skeleton consisting predominantly of trabecular bone. Findings on trabecular parameters in the distal femur metaphysis were in full agreement with the histomorphometric data. In the WTs, 5/6 Ntx coincided with increased bone turnover, as indicated by an increased eroded perimeter and increase (although not significant) in bone formation rate and marrow fibrosis.

Due to the high bone mass phenotype of $SOST^{-/-}$ mice at baseline, renal osteodystrophy changes generally appeared to be more pronounced in WT mice. In other words, because of the strong bone phenotype induced by sclerostin deficiency, only subtle skeletal changes were attributable to CKD. Overall, 5/6 Ntx, in combination with a high phosphate diet, led to a genotype-independent loss of cortical bone volume and density. Noteworthy, sclerostin deficiency did not prevent uremia-induced cortical bone loss and trabecularization in the appendicular and axial skeleton. Moreover, CKD aggravated the effects of sclerostin deficiency on bone, as indicated by more pronounced fibrosis and bone resorption.

We acknowledge several limitations of our experimental set-up: as already discussed, we did not induce overt calcification pressure in the two uremic groups. Moreover, the generalizability of our findings is incomplete since we analyzed only female animals. In general, sexual dimorphism is present in the skeleton, but here, female mice are known to be more susceptible to vascular calcification and therefore are the better models in regard to calcification experiments. At 35 weeks, the mice are quite old, which is a disadvantage in terms of assessment of bone turnover; however, aiming at a renal osteodystrophy model justifies surgery in older mice. We failed to induce a loss of trabecular bone in CKD animals versus animals with normal renal function neither in the WT's nor in the $SOST^{-/-}$ mice, despite the increased eroded perimeter and osteoclast perimeter and in the absence of an increased osteoid area and osteoblast perimeter. Although, the net result of severe secondary hyperparathyroidism in uremia is accelerated bone resorption, there is no general rule as to whether mild hyperparathyroidism and osteitis fibrosa, which both are characterized by a high bone turnover should go along with bone loss.

Lastly, complete deficiency of sclerostin is not a perfect model for human bone-vascular disease with more subtle derangements of sclerostin metabolism, and, moreover, human CKD-MBD is often complicated by interfering and aggravating comorbidities such as diabetes.

In summary, the dominance of the high bone mass phenotype in $SOST^{-/-}$ mice is preserved also after the induction of renal osteodystrophy.

Disclosures

NK, AV, ADM, AD, GJB, AH, PE, JF, NM, AB, RK, DW, PCDH and VMB declare no conflict of interest within this manuscript. IK and MK are employees of Novartis.

Figure Legends

Figure 1: Urea, creatinine and PTH in serum at the end of the experiment 12 weeks after induction of renal failure; a) urea b) creatinine c) and PTH increase significantly in serum after 5/6 nephrectomy (Ntx) (**: $p < 0.001$). For the three parameters, no differences are seen between WT and $SOST^{-/-}$ mice ($n = 5-12$).

Figure 2: FGF23 measured in serum at the end of the experiment, 12 weeks after induction of renal failure. FGF23 increases significantly in serum after 5/6 Ntx (**: $p < 0.001$) ($n = 5-12$).

Figure 3: Von Kossa staining of the heart (aortic root, 50x) in WT and $SOST^{-/-}$ mice 12 weeks after 5/6 Ntx or sham surgery. Calcifications are present in all groups. Arrows indicate localization of heart valve calcifications, which were not different between the groups

Figure 4: Total tissue calcium content of a) kidney b) carotid arteries c) myocardium in WT and $SOST^{-/-}$ mice 12 weeks after 5/6 Ntx or sham surgery. The high variability in kidney calcifications results in the absence of statistically significant differences between sham and Ntx groups (*: $p < 0.05$) ($n = 5-12$).

Figure 5: Relative expression of *Cbfa1* mRNA in aorta from WT and $SOST^{-/-}$ mice 12 weeks after 5/6 Ntx or sham surgery, measured by RT-PCR. 5/6 Ntx induced a marked increase in *Cbfa1* expression in WT animals, which was completely blunted in the $SOST^{-/-}$ mice (*: $p < 0.05$) ($n = 5-12$).

Figure 6: Bone histomorphometric analysis of femora obtained 12 weeks post 5/6 Ntx or sham surgery of WT and $SOST^{-/-}$ mice (*: $p < 0.05$; **: $p < 0.001$). a) Bone area/Tissue area

(B.Ar/T.Ar) b) Osteoid area/Bone area (O.Ar/B.Ar) c) Eroded perimeter/Bone perimeter (E.Pm/B.Pm)(d) Mineralized area/Tissue Area (Md.Ar/T.Ar) e) Osteoblast perimeter/Bone perimeter (Ob.Pm/B.Pm) f) Osteoclast perimeter/Bone perimeter (Oc.Pm/B.Pm) g) Trabecular thickness (Tb.Th) h) Trabecular number (TbN) i) Fibrosis; (n = 7-12)

Figure 7: Bone formation rate (BFR) and mineral apposition rate (MAR) measured in the entire width of the trabecular femora sample obtained 12 weeks post 5/6 Ntx or sham surgery of WT and $SOST^{-/-}$ mice (*: $p < 0.05$; **: $p < 0.001$). (n = 7-12)

Figure 8: Goldner-stained femoral sections of WT and $SOST^{-/-}$ mice 12 weeks post 5/6 Ntx or sham surgery. (n = 7-12)

Figure 9: Micro CT analysis of a) femur distal metaphysis and b) femur diaphysis and c) lumbar spine in WT and $SOST^{-/-}$ 12 weeks after sham surgery and 5/6 Ntx. BV/TV = bone volume/ tissue volume; Cn.BMD = cancellous bone minerals density; Ct.BMD = cortical bone mineral density; Ct.th = cortical thickness.

Lumbar spine- cancellous BMD: WT +85%, $SOST^{-/-}$ +24%

Distal femur metaphysis- cancellous BMD: WT +142%, $SOST^{-/-}$ +2%

(n = 7-12)

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ACCEPTED MANUSCRIPT

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Highlights

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1. Investigation of the specific phenotype of renal osteodystrophy in SOST-knockout mice with severe renal hyperparathyroidism
2. The loss of cortical bone volume and density associated with chronic renal failure is genotype independent
3. Vascular calcification was absent in SOST-deficient mice with chronic renal failure
4. The high bone mass phenotype of sclerostin-deficiency allowed only low-grade development of typical renal osteodystrophy